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13. SUPPLEMENTARY NOTES

14. ABSTRACT For efficient therapy of solid tumors such as ovarian cancer, two obstacles need to be overcome before the therapeutic potential of virotherapy could be fully materialized. Firstly, the potency of oncolytic HSVs needs to be improved. During the first two years of this funded project, we have demonstrated that incorporation of cell-membrane fusion activity into an oncolytic HSV could significantly and safely increase the antitumor potency of the virus. Secondly, host's antiviral immunity is likely to be a major obstacle for successful administration of an oncolytic virus. We proposed in the third year of this project to develop strategies to overcome this potential problem. Our hypothesis is that the ability of a fusogenic oncolytic HSV to induce cell membrane fusion would make the virus less vulnerable to the innate and/or acquired antiviral immunity once it had entered to the tumor cells, as it would be able to spread from cell to cell through syncytia formation. Thus, a strategy that could initially send the virus to the tumor site in the presence of host's antiviral immunity would be what was needed to overcome the initial hurdle of its delivery. Our data demonstrated that: 1) the fusogenic oncolytic HSVs have the ability to spread to surrounding tumor cells even in the presence of high concentration of antiviral immune sera, indicating its ability to evade the host's neutralizing antibodies once the virus has entered into the target cells; 2) an HSV-2-based fusogenic oncolytic HSV (FusOn-H2) has the ability to evade the host's innate antiviral immunity; 3) oncolytic HSVs could be formulated with liposomes for in vivo delivery; 4) in addition to T lymphocytes, NK cells and macrophages could also function as carrier cells for delivery of oncolytic HSVs by the recently reported "hitchhike" strategy. This represent a very efficient way of loading oncolytic virus to the carrier cells, which are otherwise nonpermissiveness to infection of oncolytic HSVs. With these progresses, we are confident that the remaining tasks of this project will be finished during the one-year no-cost-extension period.

15. SUBJECT TERMS
Oncolytic virus, advanced ovarian cancer, experimental therapy, syncytial formation

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INTRODUCTION

Ovarian cancer remains the leading cause of death in patients with gynecological malignancies in the United States. Because of ineffective screening strategies and inconspicuous early symptoms, these tumors are usually detected late in the clinical course, when metastases are present. The primary site of metastatic spread is within the peritoneal cavity. Despite improved surgical techniques and chemotherapy, the mortality rate for ovarian cancer has remained largely unchanged over the last decade (1, 2), mandating the development of new treatment strategies.

Replication-selective viruses afford a promising treatment for malignant solid tumors. Oncolytic viruses derived from herpes simplex virus (HSV) were initially designed and constructed for the treatment of brain tumors (3, 4). Subsequently, they have proved effective against a variety of other human solid tumors, including ovarian cancer (5, 6). However, for efficient therapy of solid tumors such as ovarian cancer, two obstacles need to be overcome before the therapeutic potential of virotherapy could be fully materialized. Firstly, the potency of oncolytic HSVs needs to be improved. During the first two years of this funded project, we have demonstrated that incorporation of cell-membrane fusion activity into an oncolytic HSV could significantly and safely increase the antitumor potency of the virus. Secondly, host's antiviral immunity is likely to be a major obstacle for successful administration of an oncolytic virus. We proposed in the third year of this project to develop strategies to overcome this potential problem. Our hypothesis is that the ability of a fusogenic oncolytic HSV to induce cell membrane fusion would make the virus less vulnerable to the innate and/or acquired antiviral immunity once it had entered to the tumor cells, as it would be able to spread from cell to cell through syncytia formation afterwards. Thus, a strategy that could initially send the virus to the tumor site in the presence of host's antiviral immunity would be what was needed to overcome the initial hurdle of its delivery. Specifically, we initially conducted experiments to examine the ability of fusogenic oncolytic HSVs to evade host's innate and acquired antiviral immunity. We also investigated the feasibility of delivering oncolytic HSVs by either formulating them with liposomes or loading them onto carrier cells.

BODY

1. Anti-HSV immune serum can't prevent spread of fusogenic oncolytic HSV in vitro.

To compare the effects of anti-HSV immune serum on the spread of fusogenic and non-fusogenic oncolytic HSV in vitro, we initially infected tumor cells (in 12-well plates) with either Synco-2D or Baco-1 at 0.01 pfu/cell for 1 h. Then, medium containing pooled mouse anti-HSV sera at 1: 50 dilution, which completely blocked Baco-1 infectivity on Vero cells, was added to the wells. As shown in Fig. 1, inclusion of the immune sera had minimal effect on syncytial formation by Synco-2D, but greatly inhibited the spread of non-fusogenic oncolytic HSV. Forty-eight h after virus infection, the cells were rinsed twice with PBS and harvested. The cell-associated virus was released by sonication. The virus was titrated by plaque assay. The result (averaged figure from two parallel experiments as shown in figure 1) showed that the presence of immune sera only

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slightly decreased the yield of cell-associated Synco-2D, but significantly reduced the production of Baco-1. These findings support the underlying hypothesis that the antitumor activity of fusogenic oncolytic HSV is less affected by antiviral immunity when compared to a conventional oncolytic HSV once the virus has entered into target cells.
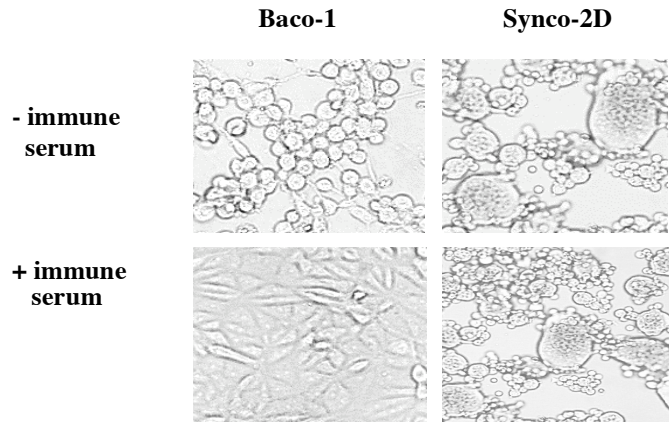

Fig. 1. Effect of anti-HSV immune sera on virus propagation and production. The photos on the left show the phenotypes and the table above gives the virus yields in tumor cells, with or without anti-HSV sera in the medium.

Table 1. Virus production in the presence of immune serum

	Virus yield	
	Baco-1	Synco-2D
No immune serum	$2.2(\pm0.2)X10^6$	$4.5(\pm1.2)X10^7$
With immune serum	$1.4(\pm0.4)X10^4$	$2.3(\pm1.3)X10^7$

2. Fusogenic oncolytic HSV can also evade the innate antiviral immunity

We also tested the ability of fusogenic oncolytic HSVs to evade host's innate immunity, which is the first line of defense against infection by a microorganism such as HSV. It has been shown that innate immunity plays a crucial role in controlling HSV infection (7-9). Consequently, it has been convincingly demonstrated that strategies to block or modify this arm of the immune system can enhance the antitumor effects of an HSV-1-derived oncolytic virus (10, 11). The inhibitory effect from antiviral immunity is probably part of the reason that although oncolytic viruses can rapidly spread in cultured tumor cells, their ability to spread within a solid tumor in vivo is often limited (12). The fact that oncolytic HSVs are rapidly cleared (within a few days) in naïve mice before the development of acquired antiviral immunity further reinforces the important role of innate immunity in restricting the replication of the virus. In this experiment, we used a fusogenic oncolytic HSV that was constructed from type 2 HSV (HSV-2) (13). We compared it with a nonfusogenic oncolytic HSV Baco-1 for their replication efficiency in the presence of bone-marrow-derived macrophages. The results showed that the replication of Baco-1 in tumor cells was significantly affected by the macrophages while the presence of the same amount of macrophages had little effect on the replication of FusOn-H2 (Fig. 2).
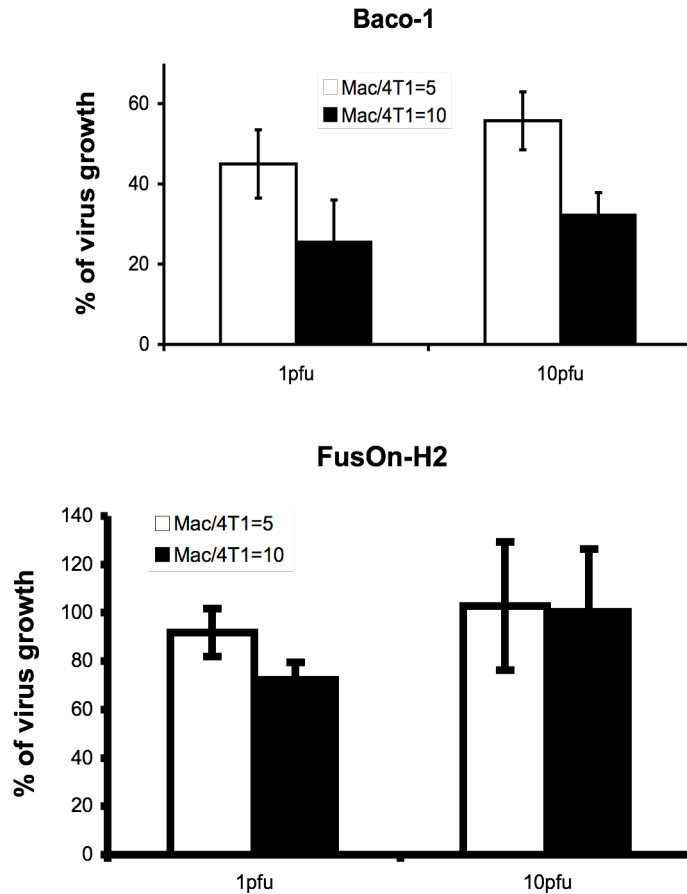
Baco-1

FusOn-H2

Fig. 2. Virus replication capability in the presence of macrophages. Macrophages were derived from freshly harvested murine bone marrow and were mixed with tumor cells that had been infected with the indicated oncolytic HSVs (at the dose of either 1 or 10 pfu/cell) at different ratios (5:1 or 10:1). Cells were harvested at 48 h later and the virus yield was determined by plaque assay. The percentage of virus growth was calculated by dividing the total virus yield from the tumor cells mixed with macrophages with the total virus from tumor cells without mixing with macrophages.

3. Liposome formulation of oncolytic HSVs. Next we tested if formulating oncolytic HSVs with liposomes could evade the host's humoral antiviral immunity. We initially tested the condition of liposome formulation of oncolytic HSVs. We prepared the virus in 3 different forms: 1) DNA form of viral genome; 2) intact viral particles; 3) de-enveloped viral capsid. We then formulated these different forms of virus preparations with liposomes and examined their infectivity in vitro by counting the plaques after adding them onto Vero cell monolayers. The results showed that viral DNA formulated with liposomes gave the highest number of plaques. Viral capsids formulated with liposome gave the second best result. The intact viral particles formulated with liposome produced the lowest number of viral plaques, possibly because the intact viral particles already contain an outside lipid membrane, which may have prevented liposome formulation.

4. Delivery of oncolytic HSVs by carrier cells. We also tested if T-lymphocytes could act as a carrier for oncolytic HSV delivery. As T lymphocytes can freely circulate through the vascular system and can infiltrate to the metastatic tumors, once loaded with the oncolytic HSVs, they may be able to send the virus selectively to the tumor sites.

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Furthermore, the cell membrane may function as a protective shield to protect the virus from neutralizing antibodies. Human or murine T cells were infected with Baco-1, which carries the green fluorescent protein (GFP) marker gene in its viral genome and its infectivity can thus be easily identified. The results showed either human or murine T lymphocytes were resistant to infection by oncolytic HSVs. Even at a multiplicity (MOI) of 10, less than 5% of cells showed GFP expression. We then examined NK cells and macrophages, another two cell types that are major components of circulating blood. In addition, both NK cells and macrophages have the ability to infiltrate to tumor tissues. We initially performed a similar in vitro experiment as described above to test the infectivity of the oncolytic Baco-1 on these cells. The results showed that both NK cells and macrophages from human and murine origins were also resistant to infection by Baco-1. At an MOI of 5, less than 10% of cells showed GFP expression.

Recently it has been reported that retrovirus can adhere nonspecifically, or 'hitchhike', to the surface of cytotoxic t lymphocytes (CTLs). CTLs hitchhiked with a retroviral vector can then move to tumor sites and "hand off" the viruses to tumor cells to initiate virus infection (14). We thus tested this strategy to determine if oncolytic HSV could hitchhike to carrier cells that were found to be non-permissive to infection of HSV vectors in our previous experiments. We incubated either NK cells or macrophages of both human and murine origins ($1X10^5$) with $5X10^5$ plaque-forming-units (pfu) of oncolytic HSV Baco-1 for 1 h. The cells were then gently washed 2 times with PBS and were added to Vero cell monolayers. Viral plaques were examined 48 h later. The results showed that Baco-1 was efficiently loaded to the carrier cells in this way and was then successfully "handed off" to the testing cell monolayers. Since more than $1X10^5$ plaques were obtained from each preparation (with $1X10^5$ carrier cells), it was concluded that approximately 100% of the carrier cells were able to hand off the oncolytic virus. These results indicate that this hitchhike strategy may be a useful way for carrier cell-medicated delivery of oncolytic HSVs for the treatment of metastatic prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS

The following accomplishments have been achieved during the past 12 months:

- Our data demonstrated that the fusogenic oncolytic HSVs have the ability to spread to surrounding tumor cells even in the presence of high concentration of antiviral immune sera, indicating the ability of this unique type of oncolytic HSV to evade the host's acquired antiviral immunity once the virus has entered into the target cells.
- The HSV-2-based fusogenic oncolytic HSV (FusOn-H2) also has the ability to evade the host's innate antiviral immunity. This will likely further enhance the ability of the virus to destroy tumor cells in vivo.
- Oncolytic HSVs could be formulated with liposomes for in vivo delivery. Especially, preparation of oncolytic HSVs in the DNA or de-enveloped capsid form could be more efficiently formulated with liposomes than the intact viral particles. Thus, these two forms of viral preparation will be used in the future in vivo studies.
- Our data demonstrate that, in addition to T lymphocytes, NK cells and macrophages could also function as carrier cells for delivery of oncolytic HSVs by the recently reported "Hitchhike" strategy. This will likely represent a very efficient way of loading oncolytic virus to the carrier cells, which are otherwise non-permissive to infection of oncolytic HSVs.

REPORTABLE OUTCOMES
> • A manuscript reporting the ability of fusogenic oncolytic HSVs to evade host's innate antiviral immunity is being drafted.
> • On March 2, 2006, Dr. Zhang was invited to give a seminar presentation to the clinical oncologists in the Texas Children's Hospital. Title of presentation: A novel oncolytic virus for therapy of solid tumors.

CONCLUSIONS

Some steady progresses have been made on demonstrating the ability of fusogenic oncolytic HSVs to evade host's innate and acquired antiviral immunity and at developing novel strategies to facilitate the oncolytic HSVs to evade the host's anti-HSV immunity. In particular, our data demonstrated the capability of fusogenic oncolytic HSVs to replicate and to spread within tumor cells in the presence of neutralizing antibodies or freshly isolated macrophages. Our data also demonstrate the "proof of principle" of using alternative ways to deliver oncolytic HSVs to tumor tissues. Especially, our data demonstrate that the recently reported "hitchhike" strategy is an ideal way of attaching oncolytic HSVs to carrier cells that are otherwise non-infectable by HSV, thus overcoming a major hurdle of this project. With these progresses, we are confident that the remaining tasks of this project will be finished during the one-year no-cost-extension period.

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